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4. Title of the invention

Height-related Gene

5. Name of your agent (if you have one)

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Height-related Gene

The sex-related height difference in humans is thought to be caused mainly by two components: first, a hormonal component determined by the sex dimorphism of bioactive gonadal steroids and second, a genetic component attributed to a Y-specific growth gene, termed GCY (Tanner, et al. 1966; Smith, et al. 1985; Ogata and Matsuo, 1992). Despite extensive mapping attempts for this gene on the human Y chromosome (Ogata, et al. 1995, Salo, et al. 1995, Rousseaux-Prevost, et al. 1996, De Rosa, et al. 1997), its precise position remains unknown. Recent evidence shows that inappropriate cytogenetic methodology in the characterization of Y-chromosomal terminal deletions has brought about some of the difficulties in elucidating the GCY-critical region. In order to overcome these problems, the inventors have considered only patients presenting *de novo* interstitial deletions for the GCY analysis on the Y chromosome (Kirsch, et al. 2000). This approach allows the assignment of GCY to a particular chromosomal interval without excluding the presence of X0-mosaicism and/or i(Yp) and idic(Yq11) chromosomes in patients with terminal deletions.

The direct comparison of overlapping interstitial deletions in seven adult males with normal height, one male with borderline height, and one patient with a large interstitial deletion and short stature resulted in the confirmation of the GCY critical interval between markers DYZ3 and DYS11. This region roughly encompasses 1.6-1.7 Mb of genomic DNA. To improve the resolution in the region of interest close to the centromere, the inventors have established additional new STS markers specific for this part of the chromosome using our bacterial artificial chromosome (BAC)/P1-derived artificial chromosome (PAC) contig. Molecular deletion analysis using these new Y-chromosomal STSs allowed the inventors to narrow down the critical interval to a genomic region of 700 kb.

The invention provides an isolated region of the Y chromosome between DYZ3 and DYS11 which encompasses GCY. Preferably the Y chromosome is a human Y chromosome.

The invention further provides isolated gene/pseudogene sequences which contributes the sex related height difference in humans.

The invention further encompasses proteins having the same function as GCY protein and which have greater than 65% homology, greater than 70% homology, greater than 75% homology, greater than 80% homology, greater than 85% homology, preferably greater than 90% homology, and most preferably greater than 95% homology to the GCY protein.

Experimental evidence will now be described in detail with reference to the figures in which:

Table 1 is a comparison of the adult height of patients and their siblings.

Table 2 is a table of new Y chromosomal STSs

Table 3 is the PCR/restriction digest analysis of sequence family variants in the AZFc region

Table 4 is a summary of BAC and PAC clones identified during physical map creation.

Table 5 is a summary of the genomic primers that will be used for microdeletion screening in adult males with idiopathic short stature.

Table 6 is a summary of the sequences of the isolated exon trap clones

Table 7A is a summary of primer pairs for predicted genes

7B is a summary of primer pairs specific for the Y-copy of Adlcan

Figure 1. Deletion mapping on the long arm of the human Y chromosome.

A diagram of the human Y chromosome with Yp telomere to the left and Yq telomere to the right is presented at the top. Shown below are the results of low-resolution analysis of Y-chromosomes of adult males with normal height or short stature. Along the top border, 95 Y-chromosomal STSs are listed. Except for SKY3 and SKY8 (see Table 2 for detail), all other STSs were previously reported (Vollrath et al., 1992, Jones et al., 1994, Reijo et al., 1995). Blank spaces or grey boxes indicate inferred absence or presence of markers for which assay

was not performed. Asterisks indicate markers in the respective breakpoint regions which could not be tested. In all cases where previously published data of the patients were re-investigated, the identical DNA sample used for the primary analysis was studied. (Please note that the proximal as well as the distal breakpoint of the interstitial deletion of patient #293 resides within satellite type II sequences.)

Figure 2. Sequence family variant (SFV) typing in the human DAZ locus in distal Yq11.23.

- A. Overview and amplicon structure of the human Y chromosome in the vicinity of the human DAZ cluster. Each amplicon is represented by specific bands (A, B, D, E, X). Shown above are arrows indicating the orientation of each member of an amplicon family with respect to each other. The amplicon indicated by bands X arose from a portion of chromosome 1 that was transposed to the distal end of the DAZ cluster and partially duplicated.
- B. Precise position of selected Y-specific STSs and the SFVs according to the physical map of the human Y chromosome. Marker sY157 is highlighted as it was suspected to be present in only one copy by multiplex PCR analysis (see text for detail).
- C. Summary of STS and SFV analysis in patients with Y- chromosomal rearrangements within the human DAZ cluster region. Grey boxes indicate inferred absence or presence of markers.
- D. Sequence family variant typing of SKY10 and SKY12 in genomic DNA of patient #1972. Assay is described in Table 3. Along the right are listed fragment sizes (in bp). Products are separated by electrophoresis in 3% NuSieve agarose (3:1) and visualized by ethidium bromide staining.

Figure 3. Schematic representation of the organization of the long arm pericentromeric region of the human Y chromosome

- A. Diagram showing the distribution of major tandem repeat blocks and general organization of sequence homologies. Basically, the region can be subdivided in three distinct intervals: a proximal region characterized by 5bp satellite sequences (G), a central region with high homology to chromosome 1 (O), and a distal region composed of X/Y-homologous sequences

(B). Below the precise position of the newly established and previously published STS markers in this region are illustrated. At the bottom border, the PAC/BAC contig constructed with the aid of the new STS markers is shown. Prefixes RP1, 5 indicate PAC clones and RP11 BAC clones, respectively.

B. Localization of the GCY critical interval as defined by high-resolution STS mapping in patients with short stature and normal height. Black boxes indicate the presence, white boxes the absence of the respective STS. Striped boxes depict the dosage unknown regions where the breakpoint resides.

Figure 4. Genomic properties of the minimal GCY critical region

4A. Diagram presenting an enlarged view of the GCY critical region. Within the homology-specific coloured genomic subintervals the PAC and BAC clones covering the minimal GCY critical region are shown against "Mapped PACs/BACs". Corresponding BAC clones sequenced by the Human Genome Project are shown against "Sequenced BAC clones" beneath. The bottom layer ("Genomic Primers") indicates the genomic distribution of the primer pairs used for microdeletion screening of adult males with idiopathic short stature.

4B. Summary of all potentially functional sequences within the GCY critical region. For the ease of overview, the genomic location of the sequenced BAC clones is renewed at the top layer. Shown below are the results of the gene prediction analysis. Precise position, orientation, and exon/intron structure of each of the potential transcriptional units is indicated. Promoter predictions obtained by the program FirstEF are presented beneath. The next layer summarizes in a similar fashion the properties of 4 apparent pseudogenes detected in this genomic region by BLAST homology searches. For completion, ARSF and RPS24Y are added. The bottom border presents the genomic location of the isolated exon trap clones.

Materials and Methods

Defining the GCY critical region

Selection of patients

Patients #293, JOLAR, #28, #63 and #95 have been described clinically in detail elsewhere (Sharc et al. 1990; Ma et al. 1993; Forcsta et al. 1998; Kleinman et al. 1999). Patient #0000

corresponds to case 1 in the study of Pryor et al. 1997. Patients T.M., #1947 and #1972 are phenotypically normal males suffering from idiopathic infertility. Genomic DNA samples were extracted from peripheral blood leukocytes (#28, #63, #95, Y0308, T.M., #1947, #1972) or from lymphoblastoid cell lines (#293, JOLAR). DNA isolated from peripheral blood leukocytes of normal males and females served as internal controls.

Height assessment

As all individuals are of diverse ethnic origins, height was compared to the respective national height standards (Table 1). Patients were of similar age range. When possible, special attention was given to adult height comparisons between parents and siblings. Data are summarized along with the height standard deviation score (SDS) in Table 1. To calculate the SDS, mean adult height and the standard deviation were taken from the corresponding national physical growth studies.

PCR analysis

Reactions were performed in a total volume of 50µl (75mM Tris/HCl pH9.0, 20mM $(\text{NH}_4)_2\text{SO}_4$, 0.1%(w/v) Tween20, 1.5mM MgCl_2) containing 1.0mM of each oligonucleotide primer, 100ng genomic DNA as template, 5 units of Taq DNA polymerase (Eurogentec), and each dNTP at 1mM in a thermocycler (MJ Research, Inc.) as follows: After an initial denaturation step of 95°C for 5min, samples were subjected to 30 cycles consisting of 30sec at 94°C, 30sec at 60°C and 1min at 72°C followed by a final extension step of 5min at 72°C. The Multiplex PCR was carried out as described in Henegariu et al. 1994 with minor modifications. *Alu-Alu* PCR reactions were essentially carried out as described in Nelson et al. 1991. Amplification products smaller than 1 kb were resolved on 3% NuSieve agarose/1%SeaKem GTG agarose (FMC) in 1 x TBE (0.089 M Tris-borate/0.089 M boric acid/20mM EDTA, pH 8.0). For amplification products larger than 1 kb as well as products from *Alu-Alu*-PCR, 1.5% SeaKem GTG agarose gels in 1 x TBE were used for separation.

PCR primers

Y-specific STSs, loci and PCR conditions have been described previously (Vollrath et al. 1992; Jones et al. 1994; Reijo et al. 1995). Sequences of new Y-chromosomal STSs are listed in Table 2. Y-specific STSs termed SKY were either derived from YAC, BAC and PAC end

sequences or from clone-internal sequences amplified by various combinations of *Alu* primers. Primers for the markers SKY10, 11, 12, and 13 were designed to amplify fragments spanning unique restriction sites within the genomic DAZ locus (SKY10 from RP11-487K20 (AC024067), RP11-70G12 (AC006983), RP11-141N04 (AC008272), RP11-366C06 (AC015973), RP11-560I18 (AC053522), RP11-175B09 (AL359453), SKY11 and SKY12 from RP11-245K04 (AC007965), RP11-100J21 (AC017005), RP11-506M09 (AC016752), RP11-589P14 (AC025246) and SKY13 from RP11-100J21 (AC017005), RP11-589P14 (AC025246), RP11-823D08 (AC073649), RP11-251M08 (AC010682), RP11-978G18 (AC073893)) in order to detect 'sequence family variants' (SFVs).

Restriction analysis of PCR products

PCR products were resolved on agarose gels, the appropriate gel bands cut out and the DNA isolated with GFXTM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Inc.) according to the manufacturer's protocol. Fragments amplified from SKY5 and SKY6 were digested with *TaqI* and *BsmI*, respectively. To detect SFVs at SKY10, SKY11, SKY12 and SKY13, PCR products were digested with restriction enzymes as listed in Table 3.

Sequencing of BAC/PAC/YAC end fragments

DNA from BAC/PAC clones selected for end sequencing were purified with the Nucleobond PC100 Kit (Macherey-Nagel) according to the manufacturer's instructions. End fragments were directly sequenced using the Thermosequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Pharmacia) and analyzed on a Pharmacia A.L.F. express (Amersham Pharmacia Biotech). YAC end fragments were generated with *Alu*/Vector-polymerase chain reaction and subcloned in pCR2.1 with the TOPO-TA cloning Kit (Invitrogen). Sequencing was performed as described.

Fluorescence in situ hybridization

Metaphase spreads were obtained either from primary blood samples or immortalized cell lines. Preparations were made according to standard protocols (Lichter and Cremer 1992). Cosmid and plasmid DNA was labeled by nick translation with biotin-16-dUTP (La Roche). Slides carrying metaphase spreads were kept in 70% ethanol at 4°C for one week. 200-300ng

of labeled plasmid or cosmid DNA, 20-30µg of human Cot-1 DNA (GIBCO BRL), and hybridization buffer (50% formamide, 10% dextran sulfate, and 2 x SSC, pH 7.0) were mixed, denatured for 5min at 75°C and pre-annealed for 30min at 37°C. The slides were denatured for 2 min in 70% formamide and 2 x SSC, pH7.0, at 72°C (Ried et al. 1992). The pre-annealed probe was hybridized overnight in a humidifying chamber at 37°C. Slides were washed and stained with avidin-conjugated fluorescein isothiocyanate (FITC). The signal was amplified with biotinylated anti-avidin followed by staining with avidin-FITC. For the probe all human telomeres (Oncor) the instructions supplied by the manufacturer were followed. Chromosomes were counterstained with 4',6-diamidino-2-phenylindol dihydrochloride (DAPI). Images were taken separately by using a cooled charge coupled device camera system (Photometrics, Tucson AZ, USA). A Macintosh Quadra 900 was used for camera control and digital image acquisition in the 'TIF' format using the software package Nu200 2.0 (Photometrics). Separate gray scale fluorescence images were recorded for each fluorochrome. Images were overlaid electronically and further processed using the Adobe Photoshop software.

Searching the stature gene

Microdeletion screening

Exon amplification

Shotgun subcloning of PAC clones into pSPL3B. Genomic DNA from chromosome Y specific PAC clones was partially digested with *Sau3AI*. 100ng of isolated fragments in the range of 4-10Kb were ligated with 100ng of pSPL3B that had been *BamHI* digested and dephosphorylated. The ligation reaction was transformed into supercompetent E.coli XL-1 blue cells (Stratagene) and aliquots of each transformation plated on selective medium (ampicillin). Resulting colonies were subsequently pooled for plasmid DNA isolation.

Cell culture and electroporation. COS7 cells were propagated in DME medium supplemented with 10% heat inactivated calf serum. For transfections COS7 cells in between the 5th and 15th passage were grown to about 75% confluence, trypsinized, collected by centrifugation and washed in ice-cold Dulbecco's PBS. 4×10^9 cells were then resuspended in cold 0.7ml

Dulbecco's PBS and combined in a precooled electroporation cuvette (0.4cm chamber, BioRad) with 0.1ml Dulbecco's PBS containing 15µg DNA. After 10min on ice, cells were gently resuspended, electroporated (1.2kV, 25µf) in a BioRad Gene Pulser 2 and placed on ice again. After 10min cells were transferred to a tissue culture dish (100mm) containing 10ml prewarmed, CO₂ preequilibrated culture medium.

RNA isolation, RT-PCR and cloning. Cytoplasmic RNA was isolated 72hrs post transfection (QIAGEN RNeasy Kit) and first strand synthesis was performed as recommended by the manufacturer with minor modifications: 5µg of RNA was added to a solution containing 10mM of each dNTP and 2µM of oligonucleotide SA2. The mixture was heated to 65°C for 5min and then placed on ice for at least a further minute. After adding a reaction mixture containing 10x PCR buffer (Perkin-Elmer Cetus), 25mM MgCl₂, 0.1M DTT and RNAsin (35U/µl), the reverse transcription reaction was transferred to 42°C for 2min. 1µl of SuperScript II RT (200U/µl; Gibco BRL) was then added and the reaction incubated at 42°C for 90min and 50°C for 30min. The entire cDNA synthesis reaction was then converted to double strand DNA using a limited number of PCR amplification cycles in the following 100µl reaction mixture: 1x PCR buffer (Perkin-Elmer Cetus), 1.5mM MgCl₂, 200µM dNTPs, 1µM SA2, 1µM SD6 and 2.5U Taq polymerase (Perkin-Elmer Cetus). 6 amplification cycles were used and consisted of 1min at 94°C, 1min at 60°C and 5min at 72°C. To eliminate vector-only and false positive products, 50U of BstXI (New England Biolabs) was added directly to the reactions, followed by overnight incubation at 55°C.

10µl of the digest was then used in a second PCR amplification using internal primers in the following 100µl reaction mixture: 1xPCR buffer (Perkin-Elmer Cetus), 1.5mM MgCl₂, 200µM dNTPs, 1µM (CAU)₄-SD2, 1µM (CUA)₄-SA4 and 2.5U Taq polymerase (Perkin-Elmer Cetus). 25 amplification cycles were used and consisted of 1min at 94°C, 1min at 60°C and 3min at 72°C. Products were separated by electrophoresis and fragments larger than the pure SD2/SA4 RT-PCR product excised and subcloned (CloneAmp pAMP1 System; Gibco BRL) into pAMP1 according to the manufacturer's protocol. Ligation reactions were then transformed in ultracompetent E.coli XL-2 blue (Stratagene) and plated on selective medium containing X-Gal/IPTG.

Identification of candidate exons. All white colonies were picked and transferred to 384-well microtiter plates containing selective medium and incubated overnight at 37°C. With a 384-pin transfer device 24.5x24.5cm culture plates with and without positively charged nylon membranes (Amersham) on top of them were inoculated and also incubated overnight at 37°C. Colonies grown on culture plates were pooled for plasmid preparation, colonies on nylon membranes were used for colony lifts. Plasmid inserts were excised, purified, and hybridized to nylon membranes containing EcoRI-digests of the PAC clones used as the original substrate. Highlighting bands were subsequently isolated and hybridized to colony lifts to identify candidate exons. Candidate exons were isolated and sequenced by Sequitherm EXCEL II DNA Sequencing Kit (Epicentre Technologies). Sequences were automatically analyzed and read on an ALFExpress DNA sequencer. Table 6 lists the sequences of the isolated exon trap clones.

In silico analysis

The genomic sequence was analyzed with the NIX (Hinxton Hall, Cambridge, UK) and RUMMAGE (IMB, Jena, Germany) analysis software packages. A total of 15 potential gene models were proposed and homologies to 6 known genes were found.

Results

Mapping of interstitial deletions

We studied the DNA of nine adult males which originally consulted reproduction centers about idiopathic infertility, but were otherwise generally healthy. Of the 9 males, 7 were unremarkable with respect to adult height. One patient, #293, with a height of 157cm, presented short stature (SDS -2.9) and one, Y0308, with a height of 165.5cm showed borderline height, being at the 3rd percentile of normal U.S. height standard (SDS -1.7). Adult height of his parents and siblings are in the normal range (Table 1), his brother being 20.5cm taller than the patient. Compared to his target height (178cm) and target range (169-187cm) he can be considered short. All men were ascertained solely on the basis of the occurrence of large *de novo* interstitial deletions on the Y chromosome. Only two of those patients had undergone previous chromosomal studies.

In our effort to localize the GCY locus, we focused on that part of the Y chromosome long arm, which was delimited by the boundaries of the interstitial deletions of the patients with short stature (Fig. 1). Recently, a detailed physical map of the human Y chromosome incorporating 758 ordered STSs and 199 completely sequenced BAC clones has been constructed (Tilford et al. 2001). We used a slightly modified PCR multiplex system (Henegariu et al. 1994) to test the absence or presence of 28 DNA loci from the Y chromosome long arm. In patients where sufficient DNA was available for further PCR analysis additional STSs were tested. As a result, 8 of 9 interstitial deletion breakpoints could be positioned (Fig. 1). As the deletions of patients JOLAR, #28, #63, #95, T.M., and #1947, all with normal height, overlap, most of the long arm of the Y chromosome could be excluded as a critical region for GCY.

As the distal breakpoint of the deletion of patient #1972 does not reside within the specific part of the Y chromosome long arm, the nature of the deletion (terminal or interstitial) remained unclear. There was also no overlap of his deletion with the deletions of patients #1947 and T.M. Relying solely on the results obtained by the STS-based interstitial deletion mapping strategy, one could not formally exclude the region distal to sY158 as a potential critical region for GCY. However, multiplex PCR analysis always showed a less intense amplification product for STS sY157 (a Y-derived marker in close vicinity of sY158). To address this problem, the rearranged Y chromosome of patient #1972 was investigated in more detail.

Fluorescence in situ hybridization and sequence family variant typing of patient #1972

The overall integrity of the Y chromosome from patient #1972 was demonstrated by FISH of the cosmid LLOYNC03”M”34F05 (PAR1) and LLOYNC03”M”49B02 (PAR2) as well as the Y-centromere-specific probe Y-97 and the telomere-specific probe ‘all human telomeres’ (data not shown). Being aware of the complex structural organization of the human DAZ locus (Fig. 2A), we specifically searched for sequence family variants (SFVs). To prevent misjudging sequence errors as single nucleotide differences, PCR/restriction-digestion assays were developed only from SFVs present in at least two overlapping BAC clones. The localization of these SFVs is shown in Fig. 2B. As these SFVs could represent allelic variants.

ten unrelated normal German males were typed. In all cases, the expected fragment pattern could be detected for the Y-chromosome derived sequences. In contrast, the fragment pattern deduced from the genomic sequence of the chromosome 1-derived BAC clone RP11-560I18 could not be confirmed (see Table 3 for detail). Each SFV-specific PCR/restriction digestion was compared to the presence/absence in the corresponding BAC clones.

Typing the genomic DNA of patient #1972 for all four sequence family variants (SKY10/Tsp509I, SKY11/NlaIII, SKY12/MseI, and SKY13/Cac8I + TfiI) revealed the absence of one Y-derived non-allelic sequence variant (Table 3 and Fig. 2C,D). In the case of SKY10 the distal copy is deleted. Not surprisingly, in all other typing experiments the more proximal copy of the respective SFVs was shown to be deleted.

Next, we investigated these SFVs in the two patients with the most distal breakpoints (#95 and #1947). Using genomic DNAs, we determined that both non-allelic variants of SKY11, SKY12, and SKY13 and one non-allelic variant of SKY10 were absent in patient #1947, whereas for all tested SFVs one non-allelic variant was absent in patient #95.

Taken together, these results provide evidence that the proximal breakpoint of the interstitial deletion present in the Y chromosome of patient #1972 resides within the interstitial deletion of patient #1947, thereby excluding this genomic region as a potential critical interval for GCY.

Refinement of the GCY critical interval

Based on the molecular analysis of the pericentric region of the long arm of the human Y chromosome (Williams and Tyler-Smith 1997), the physical extension of the GCY critical region as defined by the markers sY78 (DYZ3) and sY83 (DYS11) was estimated to constitute 1.6-1.7 Mb (Fig. 3A) of DNA. The most proximal 400 kb of this region consist exclusively of 5bp satellite sequences separated from the Y centromere only by *Alu* sequences. This constant part of the human Y chromosome is therefore unlikely to contain coding sequences. The remainder of the GCY critical region is composed of X/Y-homologous as well as autosomal/Y-homologous sequence blocks. At the onset of this study, only limited coverage in YAC clones was available for this region. In order to refine the GCY critical

interval and to generate gene finding substrates, it was necessary to establish a BAC/PAC-contig of this region.

We generated 25 additional markers mainly by sequencing the end fragments of BAC, PAC, and YAC clones as well as clone-internal sequences amplified by various combinations of *Alu-Alu* oligonucleotide primer pairs. Of those, only 7 turned out to be Y-specific (SKY1, SKY2, and SKY4-8) (see Table 2 for detail). The BAC and PAC clones identified during the generation of the physical map are summarized in Table 4. Meanwhile, some of these clones have been completely sequenced as they form part of a tiling path for sequencing the human Y chromosome (Tilford et al. 2001). The proximal part of the cloned region between markers sY78 and SKY6 has not been sequenced to date. A selection of clones covering the entire GCY critical region is depicted in Fig. 3.

Confirming the overlap between BAC RP11-295P22 and BAC RP11-322K23 appeared to be the most crucial step in the process of contig construction. Y-specific markers derived from the opposite end fragments of both clones were suspected to amplify identical-sized fragments from two different loci within the same 5bp satellite region. By testing several restriction enzymes known to cut frequently within 5bp satellites composed of the consensus sequence (TGGAA)_n, we developed loci-specific PCR/restriction digestion assays. Typing all BAC clones mapping to this sequence block with the appropriate PCR/restriction digestion assay allowed us to precisely position them thereby confirming their overlaps.

In order to narrow down the critical interval for the GCY gene, we tested for the presence of the newly generated STS in patients #293, Y0308, and JOLAR. These results allowed us to define a small region for the GCY gene (Fig. 3B, 4A). Direct sequence comparison showed that the sequenced BAC clones RP11-322K23, RP11-75F05, RP11-461H06, RP11-333E09, RP11-558M10, CITB-298B15, and CITB-203M13 completely cover the mapped region between Y-STSs SKY8 and sY83 (DYS11), suggesting that it encompasses roughly 700 kb. Basically, the region can be subdivided in three distinct intervals: a proximal region characterized by 5bp repeats, a central region with high homology to chromosome 1, and a distal region composed of X/Y-homologous sequences. As the most distal part of the GCY critical region (beginning with bp1 of BAC clone CITB-144J01) was already subject of extensive research during the process of characterization of the AZFa critical region and was

shown to harbour no functional gene (Sargent, et al. 1999), it was excluded from further detailed genomic DNA analysis. The most proximal part of the GCY critical region consists exclusively of satellite type 3 sequences of the 5bp consensus (TGGAA)_n and is therefore also not assumed to contain any gene. Leaving these two regions out of consideration, we were able to concentrate our efforts to a smaller interval of 420 kb of DNA. Large-scale sequence comparisons performed by the Advanced PipMaker software showed no integration of Y-specific sequences into the chromosome 1 and/or chromosome X-homologous regions. We have also established new Y-specific markers scattered uniformly across the entire 420Kb of DNA (Tab 5).

Transcriptional potential of the GCY critical region

To identify transcriptional units within the smaller 420 kb region, we performed exon amplification on five PAC clones (RP1-148J07, RP5-1160A12, RP1-301P22, RP4-532I07, RP1-114A11). Nine positive clones were isolated (Tab. 6). Direct sequence comparison with BAC clones showed that two clones were composed of two exons, respectively. None of the clones shared homologies with a known gene or Y-specific ESTs.

At the same time, we performed *in silico* analysis with the BAC clone sequences provided by the Human Genome Project. By the use of several gene prediction programs, a total of 15 gene models were proposed. We established Y-specific primer pairs for all predicted genes (Table 7a). Furthermore, four apparent pseudogenes (KIAA1470P, ASSP6Y, ADLP, and THC604695P) were identified by the BLAST alignment tool. To identify potential promoter regions, sequences were submitted to the FirstEF program. Extracted data are summarized in Fig. 4B.

The most prominent candidate gene so far seemed to be the Y-copy of Adlcan, as its X-homologous counterpart was shown to be upregulated in osteoarthritic tissue. Additionally, a gene model was proposed for the Y-specific copy (cfl). Direct sequence comparison of the X-derived transcript with Y-derived BAC clones revealed no conservation of splice sites and a lack of exons 3 and 4 on the human Y chromosome. Nevertheless, splicing sites may have experienced slight shifts on the human Y and exons 3 and 4 may not be essential for its Y-specific function. A BLAST homology search against the EST database showed that all Adlcan-derived ESTs correspond to its X-chromosomal copy. RT-PCR showed no signs of

expression of the Y-derived copy so far in 16 polyA⁺-RNAs tested. Ubiquitous expression was however detected of the X-derived copy with exception of all tested neurological tissues. This may indicate that the Y-copy of Adican represents a pseudogene. Alternatively, the expression profile of the Y-gene/pseudogene may be restricted in time and space and was not detectable due to these reasons. A summary of primer pairs specific for the Y-copy of Adican is given in Table 7A.

Discussion

Since the issue on the existence of a Y-specific growth gene (GCY) was first raised, there have been several attempts to define its precise location. Whereas initial studies unanimously pointed towards a common region of the Y chromosome long arm (Salo et al. 1995), more recent investigations have led to the identification of two non-overlapping critical intervals (Rousseaux-Prevost et al. 1996, Ogata et al. 1995, De Rosa et al. 1997). FISH analyses resolved this apparent contradiction by presenting clear evidence that the patient materials used in these initial investigations contained 45,X0 cells and/or i(Yp) or idic (Yq11) chromosomes (Kirsch et al. 2000). Both genetic parameters influence the adult height of a given individual, thereby rendering it impossible to predict whether such patients have lost GCY or not. Studies with patients carrying *de novo* interstitial deletions are, therefore, much better suited to address the problem of GCY localization.

In the course of winnowing the literature for patients with small interstitial deletions, in particular close to the centromere, it became clear that those patients are very rare. This prompted us to extend our search for patients carrying large *de novo* interstitial deletions, irrespective of their actual adult height. We examined 9 adult patients, 7 of whom presented normal height. Furthermore, we could show overlapping deletions, thereby excluding GCY to reside between the Y-specific marker DYS11 and the pseudoautosomal region 2 (PAR2). Two patients, #293 and Y0308, presented interstitial deletions enabling the restriction of the GCY critical region to approximately 700 kb of DNA. This region is therefore predicted to harbour one or more genes required for normal human growth.

All 9 patients studied share infertility as a common phenotype, which is in agreement with their large Yq deletions. Despite extensive routine screening of infertile males in reproduction centers, only two patients were found to present borderline/short stature in combination with a confirmed large *de novo* deletion. We therefore conclude that cytogenetically detectable *de novo* deletions enclosing the GCY gene are rare events. In addition, the adult height reduction of 6-8 cm attributed to the Y-specific growth gene (Ogata and Matsuo 1992) does not necessarily result in the diagnosis of short stature in all affected males. Sex-related adult height difference is determined by the level of bioactive gonadal steroids and the Y-specific growth gene. Parameters such as nutrition, infectious diseases and secular trend are further components influencing the adult height of a given individual. In particular, the mid-parental height contributes to the evaluation of growth reduction.

In summary, our data localizes GCY to a critical interval marked by the Y-derived markers SKY8 and sY83 (DYS11). This 700 kb interval, recently sequenced by the Human Genome Project (Tilford et al. 2001), does not contain any known gene or any Y-specific ESTs. Different reasons such as unusual gene structures, e.g. genes consisting of only one exon, the lack of homology to any identified gene, and spatially or temporally restricted gene expression patterns could account for this phenomenon.

The human genomic PAC and BAC libraries used in this work were constructed at the RPCI in Buffalo, NY. Clones isolated from these libraries were purchased from the same institution.

Table 1 Adult height comparison of patients and their siblings

Case	Country of origin	Height of patient (cm) and standard deviation score	National height standard (cm)	Heights of family members (cm) and standard deviation score
#293	U.S.A.	157 (SDS -2.9) short	176.9 (SD 6.8)	(F) 170 (M) normal (B) normal
Y0308	U.S.A.	165.5 (SDS -1.7) borderline (short?)	176.9 (SD 6.8)	(F) 170 (M) 168 (B) 188 (SDS +1.7) (S) 170 (SDS -0.4)
JOLAR	United Kingdom	168 (SDS -1.0) normal	174.7 (SD 6.7)	(F) normal (M) normal (B) normal
#28	Italy	175 (SDS -0.3) normal	176.7 (SD 6.5)	(F) normal (M) normal
#63	Ethiopia	170 (SDS +0.3) normal	168.0 (SD 7.4)	(F) normal (M) normal
#95	Israel	185 (SDS +1.4) normal	175.6 (SD 6.8)	(F) normal (M) normal
T.M.	Belgium	182 (SDS +1.3) normal	173.5 (SD 6.7)	(F) normal (M) normal
#1947	Germany	175 (SDS -0.8) normal	179.9 (SD 6.4)	(F) normal (M) normal
#1972	Germany	181 (SDS +0.2) normal	179.9 (SD 6.4)	175 (F) 165 (M) 172 (S) (SDS +1.0)

The standard deviation score (SDS) was calculated based on the equation: $SDS = (X-M)/SD$, where X is an individual's adult height and M and SD are the mean adult height and the ± 1 standard deviation of the normal population, respectively.

(M) mother, (F) father, (S) sister, (B) brother, (NA) not available.

Table 2 Y-chromosomal STSs

STS	Left Primer	Right Primer	Product
SKY1	GGACATTTGGCTGCAGAGAT	TGGCAATGCACTCTCATCAT	255
SKY2	TCAGGACAGACAGGCTGCTA	CCTGCCACTGAGCTCCTTAC	~1700
SKY3	TTCTCCCTCATCTTCCAAGC	GCTTCCATCCATTAGCAAGG	167
SKY4	CCTTTCATTCCATTCTCTTCCA	CGCACTTTATGGACTGCAA	111
SKY5*	CCCTCGTCCATTTCTTTTGA	CCTCGAATTTAATGGATTGC	202
SKY6*	TCAATGGATGCACAGTGTGGC	TCCACTGAATTCCATTGCAC	328
SKY7	GGGAGTGCAAAGGGAAAGAT	CTTTCCATGGGGTGACATTC	223
SKY8	CCATTCATTGAGTTCATTACG	ATTGGAATGGAATCGGACAG	189
SKY9	GGCCGATGGTCAAACGTGTTA	GAAACGGGCTCTGAAATTCT	531
SKY10*	ATAAGGGGCAGGTTTGTAC	GCTACTTATTCAGTGTTTAACTGACAC	329
SKY11*	AAAGTGGGTGAAGGACATGG	TTTTTGTTTGTGGCAGGTG	469
SKY12*	TTGAGTCACTGGGGATAACTG	TATGGCCCACAATCACTTCA	216
SKY13*	GGCAGCCTAGAAAGTCTTGTT	CCCTTGGGATTTTGTCTGTT	198

Markers indicated with a * amplify DNA fragments from more than one genomic locus (see Chapter *Restriction analysis of PCR products* for detail).

Table 3 PCR/Restriction Digest Analysis of Sequence Family Variants in the AZFc

STS	Restriction enzyme	BAC clones	Fragment sizes (bp) after restriction	STS	Restriction enzyme	BAC clones	Fragment sizes (bp) after restriction
SKY10	Tsp509I	487K20	279,50	SKY12	MseI	245K04	88,57,39,32
		70G12	329			506M09	145,39,32
		560I18	329*				
SKY11	NlaIII	245K04	217,154,79,19	SKY13	Cac8I/TfiI	100J21	97,83,23
		506M09	233,221,15			589P14	175,23
						251M08	97,50,33,23

*The submitted sequence of the chromosome 1-derived BAC clone RP11-560I18 (AC053522) does not show a Tsp509I restriction site within the genomic fragment amplified by the primer pair SKY10. Restriction analysis of fragments amplified from male and female genomic DNA, from a somatic cell hybrid line containing chromosome 1 as the only chromosome of human origin and from the BAC RP11-560I18 as well shows two fragments of ~180bp and ~155bp indicating a sequence error in the complete sequence of the BAC clone.

Table 4 Summary of BAC and PAC clones identified during physical map creation

Y-STTs	Positive BACs (RPCII1)	Positive PACs (RPCII, 3-5)
sY83	not screened	83D22
sY82	not screened	83D22, 114A11, 157G08, 966C15
GY8	not screened	114A11, 168E21, 271D03, 635F21, 765H16, 806O15, 904E13, 966C15
sY81	not screened	301P22, 1079J08, 1078C20, 1160A12
14A3C*	not screened	148J07, 1136A14, 1160A12, 1196I23
sY79	75F05, 79E14, 102G24, 322K23, 417D23, 600D11, 612E10, 725I12, 863I08, 903M02, 1125H21	1149H11
SKY1	376B16, 544C11, 544M21	56A05, 85D24, 958M03
SKY2	79P12, 295P22, 376L20, 828O24, 886I11, 910C06	829H08
SKY4	75F05, 322K23, 612E10	not screened
SKY5	174I24, 271E18, 295P22, 588E18, 620J20, 632F11, 684H19, 705O19	not screened
SKY6	174I24, 271E18, 295P22, 588E18, 620J20, 632F11, 684H19, 705O19	not screened

* 14A3C is a hybridization probe previously described by Tyler-Smith et al. 1993. It detects a Y-specific HindIII-fragment of 3.5 kb and an additional autosomal fragment.

Table 5 Genomic primer pairs for microdeletion screening in adult males with idiopathic short stature

Primer sequence (5'→3')		product size	primer	genomic location*	
forward	reverse			forward	reverse
ATTTCACACCGAAACCCATT	CTCCCCCTACCAACCAACACAC	251	<u>A72</u>	72300-72318	72549-72530
AGGGCCCTCACATGATTAAA	GCGACACCAATTCTTTCAT	255	<u>A92</u>	91949-91968	92204-92185
GACATCGTGGTGTCTGTTC	CAGACGTTGTTCAAGTCTGTG	232	A111	111509-111528	111740-111721
GCACCATTAAGTGGCTTGT	TTCTCCCTTACCCCAATTTC	269	<u>A134</u>	134542-134560	134810-134790
CCAGCAGGAGTCTTGGAGTC	TGAGAGGACCACTACGGTTAGA	250	<u>A158</u>	157911-157930	158160-158140
CCAAGCATGCCCTTCCATAAG	TGCCCTTCATCTGCTTGTG	147	B17	17598-17617	17744-17725
ATCTGGGAGATGCATCAGA	TGAGTCTCTAAACCGTACACATACA	209	B37	37406-37425	37614-37591
^{b r 002} for CAATGGAAATGTTGCAGGTG	TCCTGCCCTGCTGTTAGAGT	158	<u>B59</u>	59871-59890	60028-60009
GCAAGGGTGTGCAAGTTA	TGCATATTGTCCACACATGG	360	<u>B82</u>	82128-82147	82487-82468
AAAGAGAAGGGCCCTGTGAT	CTAGGCAACAGCACTGGAAA	239	<u>B102</u>	102854-102873	103092-103073
AAAAATCCAACCTTCCCCAGTG	GCAAGAATCTGGGCTCTCAC	353	C17	17307-17326	17659-17640
CACCTGGGGAAGGCTGTGATA	CATTGTCACTCACTGCCAGGT	339	C37	37271-37290	37609-37590
CCCACTTCTTCTCCAAAGTCC	GCACCCGTTTCTCTGATCTA	139	C56	56159-56179	56297-56278
^{c r 005} rev GGGGCATATTCTACACACCAA	TGAAATGGCAAAACCTTTCAGA	495	C77	76731-76751	77225-77205
AAGAATGGGAAGGATCTCCAAGA	TCGTGTGAGAAATGATGATTC	342	C97	96759-96780	97100-97079
TGGTAGTGGGAAACTGCTCA	TGGTGTGCTAAGTGGCTGTC	144	C120	120709-120728	120852-120833
^{c r 003} rev GCTGCAGTTAGCTAAACCAAGAC	ATTCTGCCTGAACCTTCAGA	162	C142	142289-142311	142450-142431

Table 6 Sequences of isolated exon trap clones

Exon trap clones:

Name	Sequence (5'→3')	Size (bp)	Orientation
et_a_001	GGTCTTTGGCTCAACTCAGGTTCCCTCTACCTGAAATGATCCACCTTCAGAGATTGATG	61	reverse
et_a_002	CTGTGTTGCCCTCCTCGATGGGAAAAGAAACAAGCCACTAATGTCATTT (exon 1) CTGAGCATCAGGGGTCTCTTCTATGATCAAGGAAGGAGCCACTCAGGGTGATAGAGCTGCAACTCTGCTTGTCA CTCTGATAGCTCTGGGAACACTGTGCACCTCTCTGGCTGTGATGGGAAACT (exon 2)	182	reverse
et_a_003	CTTTTACATAGAATGTTAACTCCTTTTGACCTCGTGTTC	44	forward
et_a_004	AAAGTTGTAGTTCGCTCCCGGGCTGATGCTCAGAGTGTGAACTTGAGGAGCTGCGGTGACATCCTGCAGCCACAGG GAGGTGGCTCCCTCAGGGGGGATTTGCTGGCTGTGTCAACCACAGGGGACACCGGGCACAGCTTGAAGCTTGGGGACAGGG AGCTGAGAGGCAC	171	reverse
et_c_001	GATTACATGGACTACTATATTTTAAATTCCTTCTAAACTTTTCCCATTTCTGCTCAATTTTCATTCTCCAATATTTGC AAACTTAAAGTTC	93	forward
et_c_002	GCTGAACATTAATTCTTTATTTCCAGATTAGAGACTAGGATTCATGGGATTATGCATCAA	60	forward
et_c_003	GGAATCTTGAAATGGCAAACTTTCAGAAAGATGGCAGAGACTCTCCTACATATTCGTCTCAAT	68	reverse
et_c_004	ACACTGGAAGAATGTGTCTAGGCAGTCTGGGATAATAGCCTAAGTTCTAAGACATTAATCATTTGATCCCTTAATAGGC CATAGACCTCCAT (exon 1) TTCTTCCCTGTGTGCAGAGGGGTGATTTAAGGCTTTTCTACCTTAAGTTGATCAAAAGTGTATTTTCATTAAGATTAA TCTGGCAGCAGAAATGCA (exon 2)	188	reverse
et_c_005	CTTGGTTGGAAATATAGCCACCATATTTGCTGGGAAGCCACCAAGAAGTGACTGTACCAATATCCAAGGACATGA	79	forward

Table 7A Primer pairs for predicted genes

Primer pairs for predicted genes					genomic location	
forward	reverse	product size ¹	predicted gene	restriction enzyme ²	forward	reverse
GCTTGAAGTGTGAGGTGCTC	GGAGATGTGGGCTTGAGT	482	a_r_001		104600-104581	103800-103800
CTGTGGGTGCAITTAGGTGTG	CTGGTACATGCTGCCTGCT	841	a_r_002		144939-144920	144939-144920
GACCTCTTTTGAGAAAGTCAGCA	AAAGCAATGGCAACAAAGC	446	b_f_001		30214-30236	30214-30236
AGAGGGAGGAAAGAGCCATC	GTTGTACGGGCTGCAGAATC	790	b_r_001		25244-25225	25244-25225
TGAGTCCIAAACCGTACACATACA	TTCTGTGCGTGAGAACACA	122	b_r_002		37614-37591	37614-37591
TCTCTGTGGTGTGATCCTG	GCAAGAATCTGGGCTCTCAC	730	c_f_001		6243-6262	6243-6262
ATCCCTATTGCCCCCTAGA			c_f_001b		10734-10753	10734-10753
ACCTCAGGGTGCAGCTTTA	TGAGCAGTTCCCACTACCA	350	c_f_002	Bsh1236I	80230-80249	80230-80249
GCTGCAGTTAGCTAAACCAAGAC	TTCTGCAAGGCTCTGTTCT	123	c_f_003	AlwI	142289-142311	142289-142311
CACAGAAGCCAGGGATCG	GCACTCGCCCTTTCCTC	1150	c_r_001	BamHI	6361-6344	6361-6344
CAACACTGTACACCGCAACA	TTCTCCAAGTCCGATACCTG	172	c_r_002	BspMI	81022-81003	81022-81003
TGGAGACATTACACAACGTCAA	TGGTAGTGGGAAACTGCTCA	325	c_r_003	AluI	129988-129968	129988-129968
AGCTGCCTGACTTCTTGGA	CTTGCCACACCTTGATCTC	574	c_r_004	AccI	170431-170412	170431-170412
CGTGTGGAATTCCTATTGG	CCCACTTCTTCTCCAAAAGTCC	212	c_r_005	MspI	66318-66299	66318-66299

¹predicted product size in bp; ²Potential Y-derived transcript copies will be cut with the indicated restriction enzyme, potential X-derived transcripts remain uncut; ³indicates primer positions (orientation centromere to telomere) in the predicted gene containing BAC (a, b, c or d).

Table 7B Primer pairs for Y copy of Adlican

Primersequence (5'→ 3')	Direction with respect to putative transcription orientation		primer
GACTCCTGGCCTTGACTTGA	forward	45 - 64	AdlYEx1
TCTCTGTGGTGCTGATCCTG	forward	185 - 204	<u>cfl</u>
GGAGGAGCAAAAACAAGAAGAGA	forward	514 - 536	<u>cfl-117</u>
ACTGATGAGCACGGGAACC	forward	602 - 620	cfl-205
TCCATCCTGAAAGTGCCTG	forward	1190 - 1208	<u>C17c</u>
ACATGTATACATGCTGCCAA	forward	1513 - 1532	<u>C18</u>
CAGCGAAGGAAAGCACATTT	forward	2178 - 2197	AdlYEx5
GGCGACCTGAAGGGGACT	forward	2341 - 2358	cfl-1915
CTGTCCAGTCCTCAGGAAGC	forward	5090 - 5109	<u>C21</u>
GAAGCATCCACCAAAGCG	forward	5105 - 5122	<u>cfl-4679</u>
ACAGCGGGCGCTATGAGT	forward	5972 - 5989	<u>cfl-4a</u>
CAGGATCAGCACCACAGAGA	reverse	204 - 185	AdlYEx2
CTGGGGAAGTTGGATTTTCTC	reverse	580 - 560	C17b
ACCAGGTTCCCGTGCTCA	reverse	624 - 607	cfl-227
GCAAGAATCTGGGCTCTCAC	reverse	915 - 896	cfl
ACTGTGATTCCCACCGTGAT	reverse	1638 - 1619	C17c
TTGTTTTGAGGAACGCCTCT	reverse	1813 - 1794	C18
GGATGTGGGATCTGGTGAG	reverse	2505 - 2487	cfl-2079
GGGTGTAATTTTCTCCATTG	reverse	3104 - 3084	AdlYEx5
CGTCCGTTTCAGCAGTGACA	reverse	5236 - 5217	cfl-4810
CTGACGTCCGTCCTCTGC	reverse	6144 - 6127	cfl-4b
ATGGACAGTGATCCGTTTC	reverse	7159 - 7140	cfl-6453
TGAGCTGCACGATCAACCTC	reverse	7265 - 7246	cfl-6559

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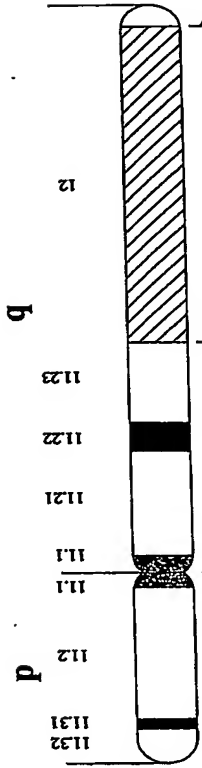
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Figure 1



STS: #Y78 #Y81 #Y83 #Y84 #Y85 #Y86 #Y87 #Y88 #Y89 #Y90 #Y91 #Y92 #Y93 #Y94 #Y95 #Y96 #Y97 #Y98 #Y99 #Y100 #Y101 #Y102 #Y103 #Y104 #Y105 #Y106 #Y107 #Y108 #Y109 #Y110 #Y111 #Y112 #Y113 #Y114 #Y115 #Y116 #Y117 #Y118 #Y119 #Y120 #Y121 #Y122 #Y123 #Y124 #Y125 #Y126 #Y127 #Y128 #Y129 #Y130 #Y131 #Y132 #Y133 #Y134 #Y135 #Y136 #Y137 #Y138 #Y139 #Y140 #Y141 #Y142 #Y143 #Y144 #Y145 #Y146 #Y147 #Y148 #Y149 #Y150 #Y151 #Y152 #Y153 #Y154 #Y155 #Y156 #Y157 #Y158 #Y159 #Y160 #Y161 #Y162 #Y163 #Y164 #Y165 #Y166 #Y167 #Y168 #Y169 #Y170 #Y171 #Y172 #Y173 #Y174 #Y175 #Y176 #Y177 #Y178 #Y179 #Y180 #Y181 #Y182 #Y183 #Y184 #Y185 #Y186 #Y187 #Y188 #Y189 #Y190 #Y191 #Y192 #Y193 #Y194 #Y195 #Y196 #Y197 #Y198 #Y199 #Y200 #Y201 #Y202 #Y203 #Y204 #Y205 #Y206 #Y207 #Y208 #Y209 #Y210 #Y211 #Y212 #Y213 #Y214 #Y215 #Y216 #Y217 #Y218 #Y219 #Y220 #Y221 #Y222 #Y223 #Y224 #Y225 #Y226 #Y227 #Y228 #Y229 #Y230 #Y231 #Y232 #Y233 #Y234 #Y235 #Y236 #Y237 #Y238 #Y239 #Y240 #Y241 #Y242 #Y243 #Y244 #Y245 #Y246 #Y247 #Y248 #Y249 #Y250 #Y251 #Y252 #Y253 #Y254 #Y255 #Y256 #Y257 #Y258 #Y259 #Y260 #Y261 #Y262 #Y263 #Y264 #Y265 #Y266 #Y267 #Y268 #Y269 #Y270 #Y271 #Y272 #Y273 #Y274 #Y275 #Y276 #Y277 #Y278 #Y279 #Y280 #Y281 #Y282 #Y283 #Y284 #Y285 #Y286 #Y287 #Y288 #Y289 #Y290 #Y291 #Y292 #Y293 #Y294 #Y295 #Y296 #Y297 #Y298 #Y299 #Y300 #Y301 #Y302 #Y303 #Y304 #Y305 #Y306 #Y307 #Y308 #Y309 #Y310 #Y311 #Y312 #Y313 #Y314 #Y315 #Y316 #Y317 #Y318 #Y319 #Y320 #Y321 #Y322 #Y323 #Y324 #Y325 #Y326 #Y327 #Y328 #Y329 #Y330 #Y331 #Y332 #Y333 #Y334 #Y335 #Y336 #Y337 #Y338 #Y339 #Y340 #Y341 #Y342 #Y343 #Y344 #Y345 #Y346 #Y347 #Y348 #Y349 #Y350 #Y351 #Y352 #Y353 #Y354 #Y355 #Y356 #Y357 #Y358 #Y359 #Y360 #Y361 #Y362 #Y363 #Y364 #Y365 #Y366 #Y367 #Y368 #Y369 #Y370 #Y371 #Y372 #Y373 #Y374 #Y375 #Y376 #Y377 #Y378 #Y379 #Y380 #Y381 #Y382 #Y383 #Y384 #Y385 #Y386 #Y387 #Y388 #Y389 #Y390 #Y391 #Y392 #Y393 #Y394 #Y395 #Y396 #Y397 #Y398 #Y399 #Y400 #Y401 #Y402 #Y403 #Y404 #Y405 #Y406 #Y407 #Y408 #Y409 #Y410 #Y411 #Y412 #Y413 #Y414 #Y415 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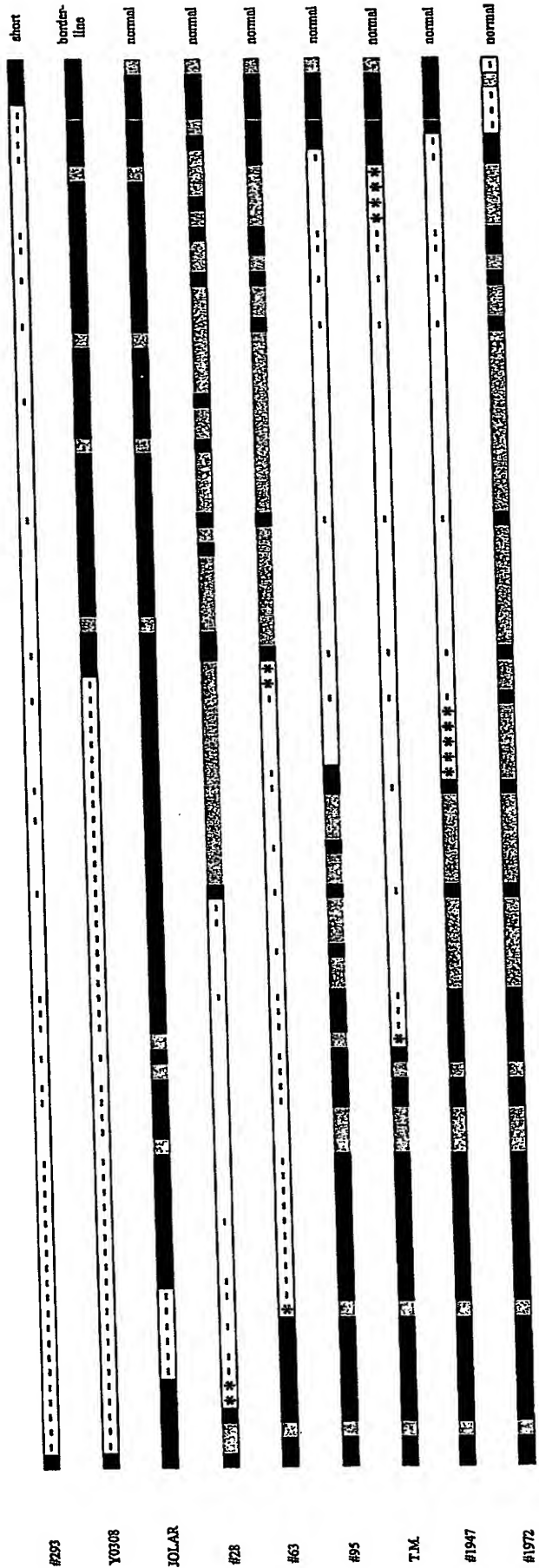
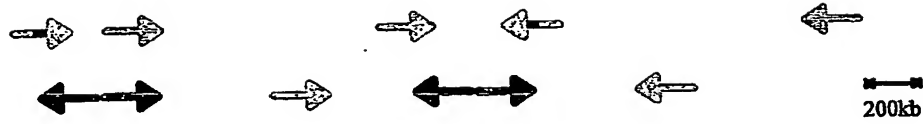
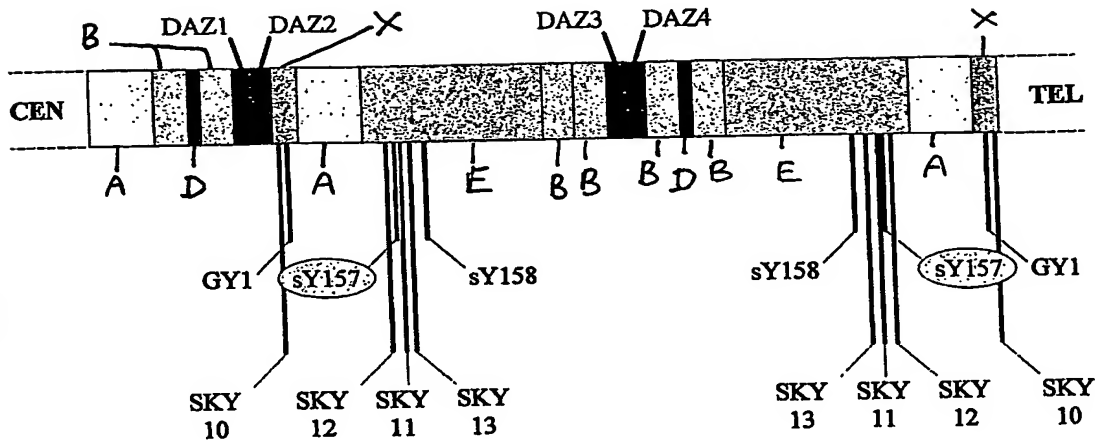


Figure 2

A



B



C

	SKY 10	GY1	SKY 12	sY157	SKY 11	SKY 13	sY158		sY158	SKY 13	SKY 11	sY157	SKY 12	GY1	SKY 10
#95	-	-	+	+	+	+	+		-	-	-	-	-	+	+
#1947	-	-	-	-	-	-	-		-	-	-	-	-	+	+
#1972	+	+	-	-	-	-	+		+	+	+	+	+	-	-

D

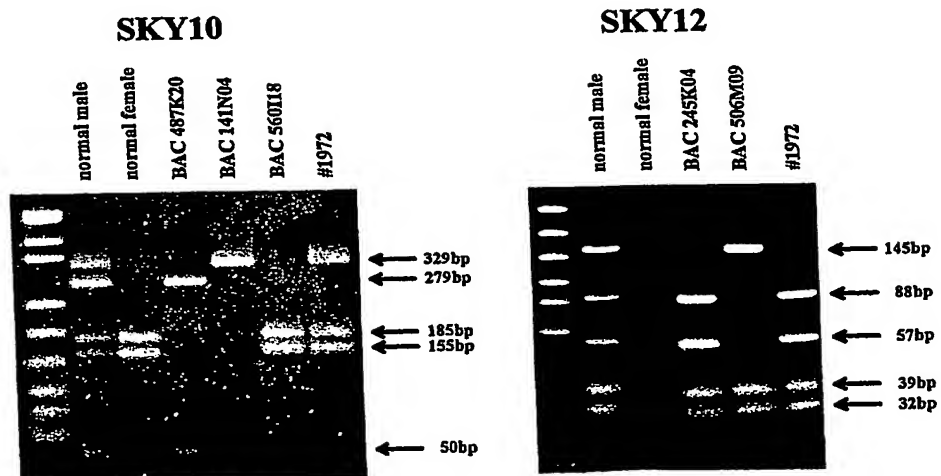


Figure 3

Homology to:

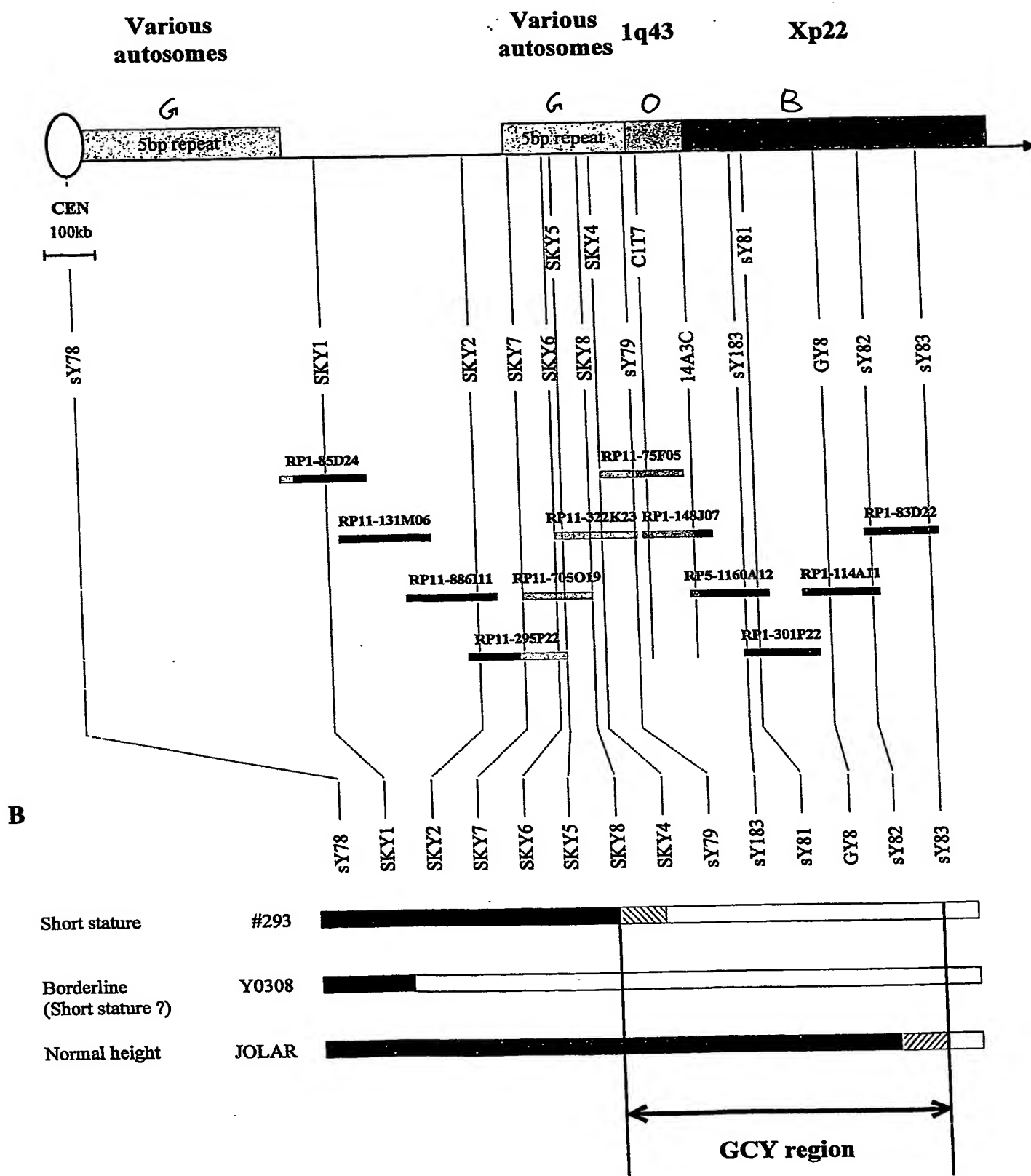
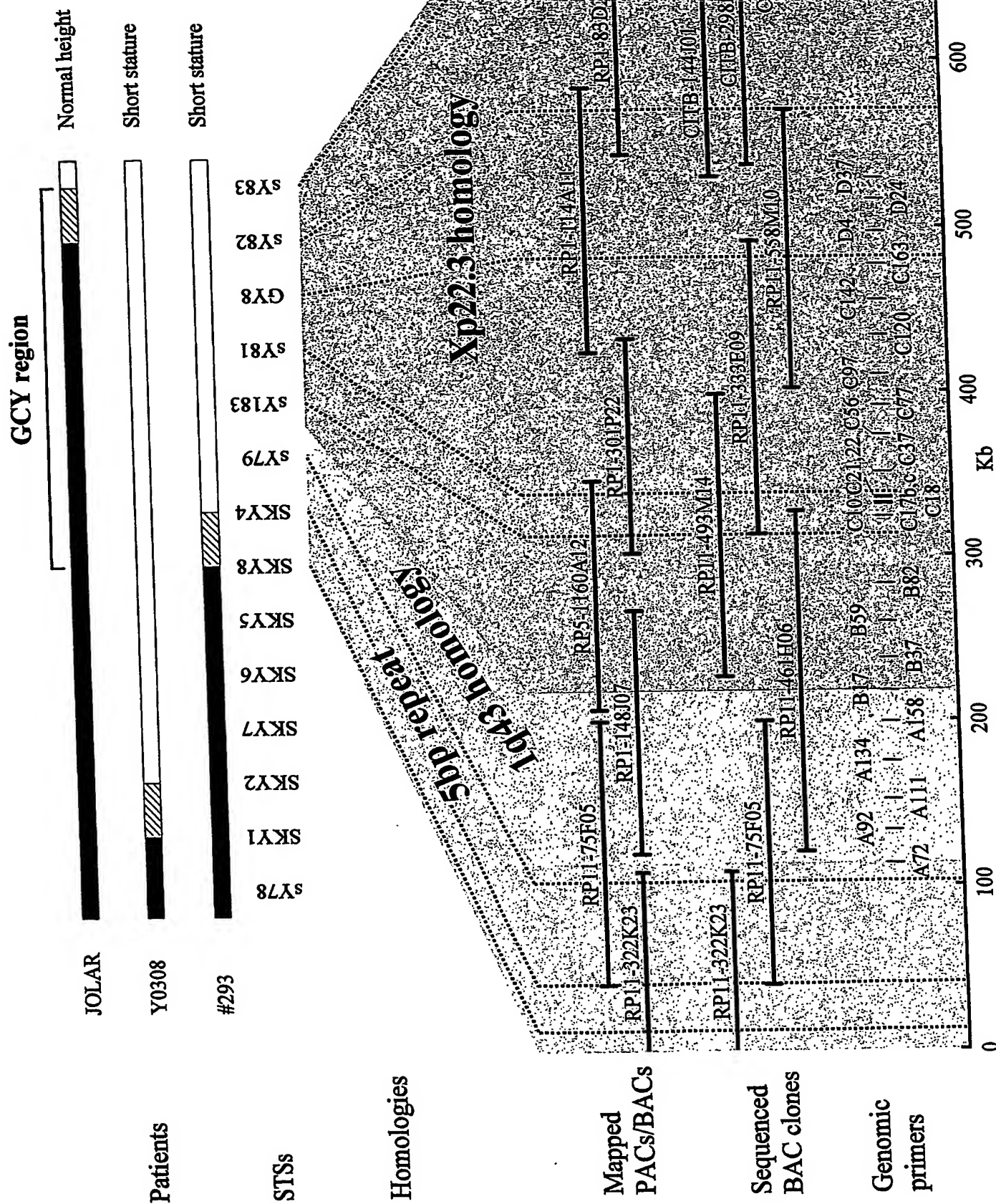
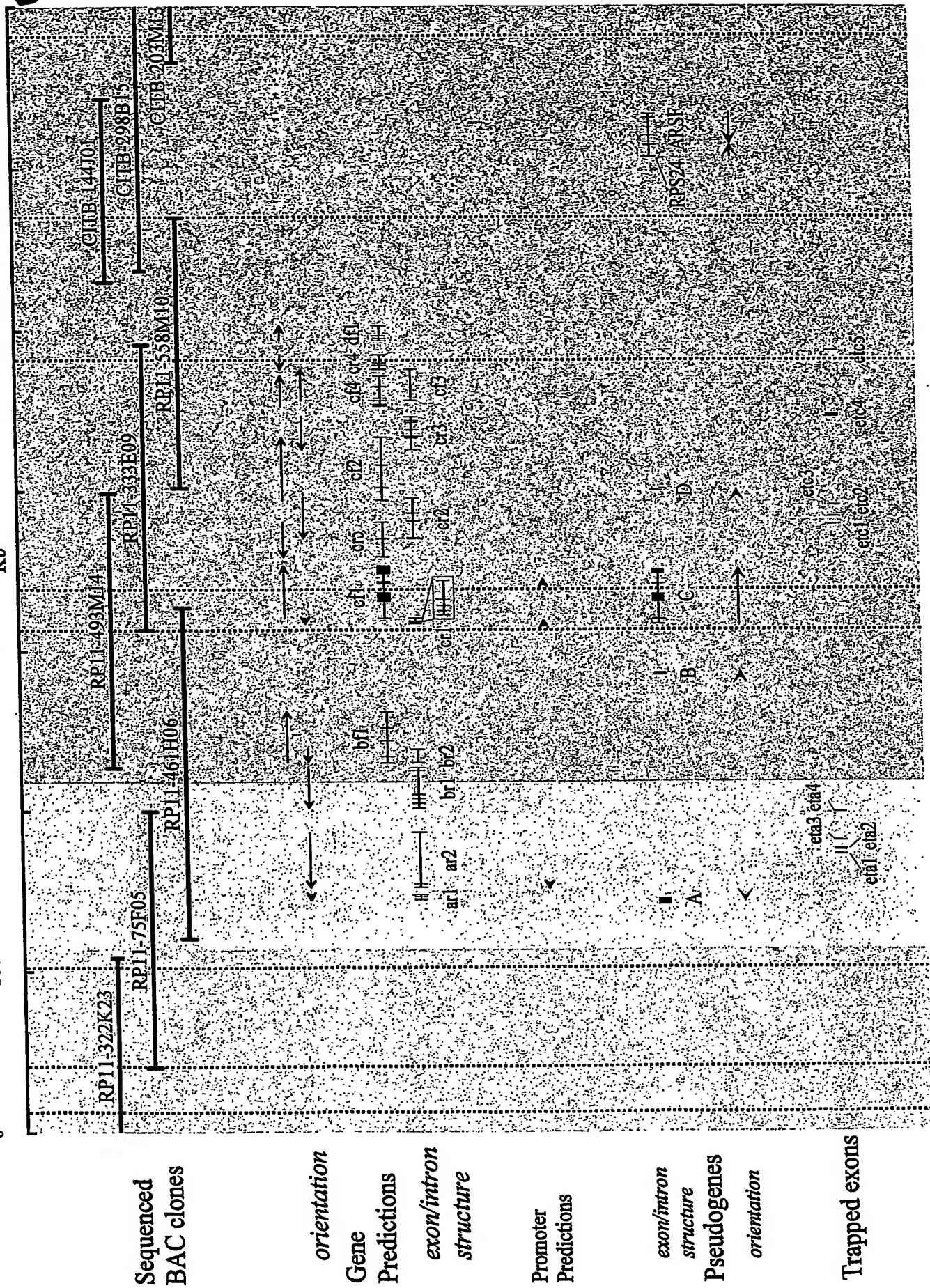


Figure 4A







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